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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
Uncontrolled proliferation of tumor cells due to failure of immune-surveillance has been linked to cancer development. Regulatory T cells (Treg) play a critical role in immune tolerance by suppressing immune responses to the body's own antigens. The tyrosine phosphatase SHP-1 is a well-known negative regulator of T cell signaling that also affects the generation of Treg cells. Interestingly, mice with decreased levels of SHP-1 protein show a high occurrence of breast cancer. The objective of the studies proposed in the concept award is to test the hypothesis that the CD4+CD25+ regulatory T cell population plays a role in the increased incidence of breast tumors we have observed in me/+ mice. During the first year of the award, we have characterized transgenic mice expressing a dominant negative mutant of SHP-1 in the T cell lineage. Analyses of these mice have demonstrated a T cell autonomous effect of SHP-1 as assessed by TCR/CD3-mediated hyper-proliferation of the mice expressing the dominant negative mutant compared to non-expressers. Moreover in preliminary data, it was observed that expressers show an increase in CD4+CD25+ Treg cells compared to non-expressers making these transgenic mice a powerful model system to directly test the hypothesis that SHP-1 deficiency promotes the development/onset of breast cancer by increasing the number of regulatory T cells.

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# Annual Report (Year 1) for Concept Award
## "Role of the Tyrosine Phosphatase SHP-1 and Regulatory T cells in Breast Cancer"

## Introduction

The failure of immune-surveillance by the body allowing uncontrolled proliferation of tumor cells has been linked to cancer development. Regulatory T cells ($T_{reg}$) play a critical role in immune tolerance to self by suppressing immune responses to autoantigens. For a variety of cancers, including breast cancer, increased $T_{reg}$ cell numbers have been reported. The tyrosine phosphatase SHP-1 is a well-recognized negative regulator of T cell signaling that also affects the generation of $T_{reg}$ cells (1). Interestingly, mice heterozygous for the *motheaten* (*me*) allele, which express decreased SHP-1 protein levels, show a high occurrence of breast cancer.
The objective of the studies proposed in the concept award is to test the hypothesis that the CD4+CD25+ regulatory T cell population plays a role in the increased incidence of breast tumors we have observed in *me*/+ mice.

## Body

As we had briefly outlined in our request for a no-cost extension of this award, over the last year, we encountered a number of unexpected difficulties in animal husbandry due to problems in the animal facility, which were beyond our control. These circumstances prevented us from analyzing *me*/+ and +/+ older mice as proposed in aim 1 of our original objectives, but instead we focused our studies on characterizing the newly generated transgenic mice that are the basis of aim 2.

The original hypothesis of aim 2 was to test whether the increased incidence of breast cancer in *me*/+ mice is specifically due to impaired SHP-1 function in the T cell lineage. In order to address this question, we have generated mice that carry dominant negative mutants of SHP-1 (C453S and D419A) using the Cre/loxP system sites (2, 3). that allows tissue-specific expression upon crossing into Cre-expressing mouse strains (Fig. 1). We have obtained 2 (C453S) and 4 (D419A) independent transgenic lines that are currently further characterized.

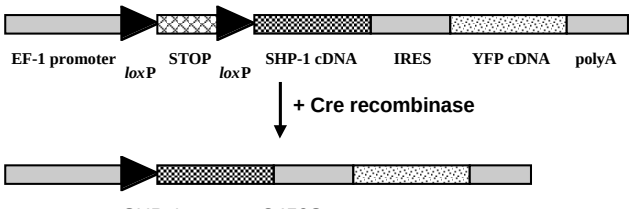


**Figure 1:** Transgenic construct to generate mice expressing wild type and dominant negative mutants of SHP-1 (C453S and D419A) in a tissue-specific manner.

Upon crossing with Lck-Cre mice, expression of the SHP-1 mutants is targeted to the T cell lineage. Analysis of the first generations of mice showed lineage-specific expression as assessed by western blotting (Fig. 2A) and flow cytometry (Fig. 2B-C).

To evaluate whether the putative dominant negative mutants for their effect on TCR-mediated signaling, we assessed TCR/CD3-driven proliferation in mice expressing dominant negative SHP-1 in the T cell lineage. In particular, TCR-induced proliferation of splenic T cells derived from SHP-1 D419A and *lck*-Cre double transgenic mice or the control mice single transgenic for either SHP-1 D419A or *lck*-Cre. As shown in Fig. 3, T cells derived from double transgenic 12-14-week old mice were hypersensitive to TCR stimulation. Interestingly, the 2-3 fold leftward shift observed in mice expressing the dominant negative SHP-1 mutant compared to controls is highly similar to the phenotype observed in SHP-1-deficient *me/me* T cells (4, 5). We are very encouraged by this finding, since it further validates that T cell-specific expression of the SHP-1 D419A mutant mimicks the *me/me* T cell phenotype. Moreover, since only the T cell lineage was affected by the loss of SHP-1 in these

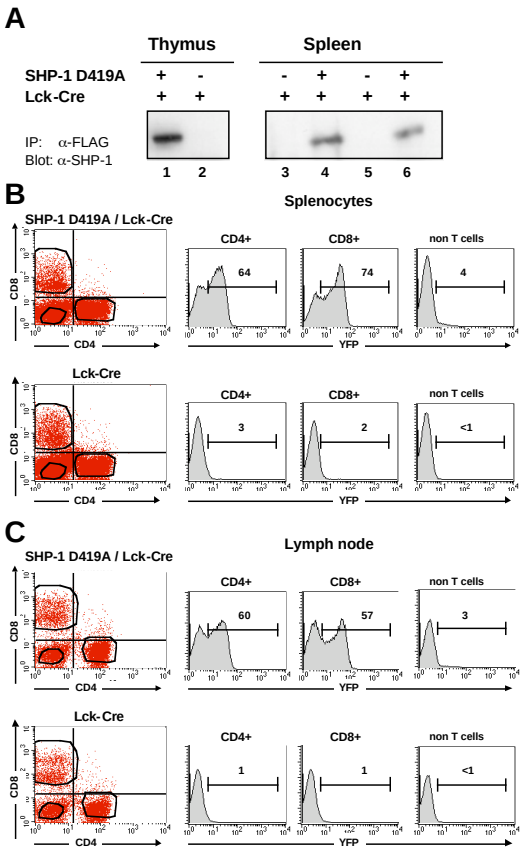


**Figure 2:** *Mice double transgenic for SHP-1 D419A and Lck-Cre express transgene.* (A) Transgenic flag-tagged SHP-1 D419A was immunoprecipitated from lysates of thymi (2 x $10^7$ cells) or spleens (lanes 3 and 4: 4 x $10^6$ and lanes 5 and 6: $10^6$ cells/IP) of mice carrying indicated genotypes followed by immunoblot with anti-SHP-1. Flow cytometric profiles shown in B and C are from mice analyzed in lanes 5 and 6. Spleens (B) and lymph nodes (C) were isolated from 8 week old mice either transgenic for SHP-1 D419A and Lck-Cre (top of A and B) or for Lck-Cre alone (bottom of A and B). Cellular suspensions were stained for flow cytometry with the indicated antibodies. Gated subpopulations were analyzed for YFP expression. Cells not positive for CD4 or CD8 are referred to as non T cells. Percentages of YFP+ cells within each subpopulation are indicated.

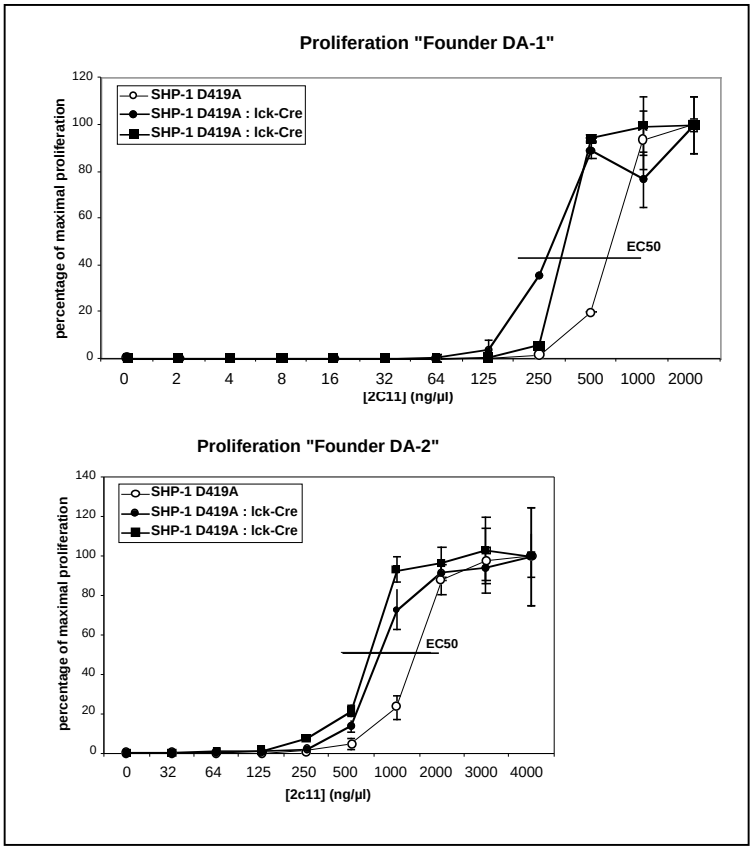


**Figure 3:** *T cells from transgenic mice expressing dominant negative mutant of SHP-1 are hyper-sensitive to TCR/CD3 stimulation.* Proliferative response of T cells derived from single (SHP-1 D419A) or double (SHP-1 D419A : *lck*-Cre) transgenic mice in response to increasing amounts of anti-CD3ε antibody (2c11). Splenocytes were enriched for T cells using negative selection via magnetic beads (Miltenyi). 5 x $10^4$ T cells were stimulated with the indicated concentrations of plate-bound anti-CD3ε. After 72 hrs., cells were pulsed with 1 µCi of [$^3$H] Thymidine for 18 hrs. [$^3$H] Thymidine incorporation was measured using a cell harvester. The data are presented as percentage of maximal incorporated counts at the highest concentration of anti-CD3ε. In general maximal incorporation was ~200.000 cpm. The two graphs represent experiments using offspring (~3 month old) from two different founder mice (DA-1 and DA-2). Error bars represent the standard deviation of the mean.

experiments, rather than the entire hematopoietic system as in *me/me* mice, it suggests a T cell-autonomous effect of SHP-1.

We then assessed whether expression of the D419A mutant solely in the T cell lineage affects the number of CD4+CD25+ Treg cells in these transgenic mice. A preliminary analysis of spleen and lymph nodes of a limited number of mice showed a promising statistically significant increase in the number of CD4+CD25+ Treg cells in mice expressing the D419A mutant compared to non-expressers (Tab. 1). We realize that these are preliminary data; however, the observed increase in CD4+CD25+ Treg cells is again comparable to our previous data obtained from *me/me* mice (1). Thus this further supports the feasibility of the proposed studies to test the hypothesis that increased numbers of Treg cells contribute to the development of breast cancer. We are currently backcrossing the transgenic mice into the BALB/c strain, since breast cancer is more often detected in this genetic background compared to the C57/B6 background the mice are currently in. Backcrossing, Treg analyses and observation for the onset of breast cancer will be the focus of this research during the second year of the concept award.

| genotype | N | CD4 T cells (%) | CD4+CD25+ (% within CD4+ population) | p value |
|---|---|---|---|---|
| **Lymph node** | | | | |
| SHP-1 D419A**-** / Lck-Cre**+** | **3** (4) | **47.0±4.3** (44.9±5.6) | **5.0±0.9** (5.7 ± 1.8) | **0.0057** (0.1364) |
| SHP-1 D419A**+** / Lck-Cre**+** | **4** | **42.2±4.5** | **7.0 ±0.2** | |
| **Spleen** | | | | |
| SHP-1 D419A**-** / Lck-Cre**+** | **3** (4) | **19.3±0.5** (18.2±2.6) | **6.9±1.3** (7.7 ±2.1) | **0.0174** (0.0949) |
| SHP-1 D419A**+** / Lck-Cre**+** | **4** | **13.7 ±1.9** | **9.9±1.0** | |

**Table 1**: CD4+CD25+ Treg cell analyses of D419A expressing and non-expressing mice. N=number of mice analyzed in each group. Initially 4 mice were analyzed in each group. However upon further analysis, one outlier was identified in the SHP-1 D419A- / Lck-Cre+ group and based on the Grubb's test for outliers, this data point was omitted for the final analysis. Data including this outlier are provided in parentheses. p values were calculated for the changes in percentages of CD4+CD25+ Treg cells within the CD4 T cell population. Statistical analyses showed a statistically significant increase in the percentage of Treg cells both in the lymph nodes as well as in the spleen of mice expressing the SHP-1 D419A mutant compared to non-expressing mice.

## Key Research Accomplishments

- Generation of transgenic mice expressing the SHP-1 D419A mutant specifically in the T cell lineage
- D419A expressers show T cell autonomous effect of SHP-1 as assessed by TCR/CD3-mediated hyper-proliferation
- D419A expressers show increased numbers of CD4+CD25+ Treg cells

## Reportable Outcomes

Not yet

## Conclusion

Based on the preliminary data obtained from the SHP-1 D419A/Lck-Cre mice, which demonstrate a T cell autonomous effect of SHP-1 as evidenced by effects on T cell proliferation and increased CD4+CD25+ Treg numbers, we conclude that the approach proposed in aim 2 (analysis of mice deficient in SHP-1activity in the T cell lineage) of our original application is feasible and is in fact better suited than the alternative approach of the original aim 1 (analysis of mice deficient in SHP-1activity in all SHP-1-expressign lineages) to directly address the hypothesis that SHP-1 deficiency promotes the development/onset of breast cancer by increasing the number of regulatory T cells. During the second year of this award, we will therefore focus our effort on this part of the original proposal.

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## Appendices

Not applicable